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Isolation and characterization of human islet stellate cell

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Abstract

Background and aims: Our previous research reported islet stellate cell (ISC) exhibiting similar appearance to classical pancreatic stellate cell (PSC) was isolated from freshly isolated rat islets, which may contribute to the islet fibrosis in type 2 diabetes mellitus (T2DM). This study was designed to determine whether human islets also contain ISC, and if so, whether human ISC is different from human PSC.

Materials and methods: Using standard explants techniques, human ISC was isolated from freshly isolated human islets, while human PSC used as a positive control was isolated from pancreatic tissue. Immunofluorescence visualization of markers for PSC (α -smooth muscle actin (α -SMA), desmin, vimentin, glial fibrillary acidic protein (GFAP)) was used to characterize the human ISC. Cell counting kit-8 (CCK-8) was used to assess the proliferation of ISC. The wound-healing assay and the transwell migration were used to assess the migration capacity of ISC. Immunofluorescence against collagen types I (col-I), collagen types III (col-III) and fibronectin (FN) was performed to identify extracellular matrix (ECM)

component synthesized by ISC. Adipogenic and osteogenic differentiation were tried to detect stem cell potential.

Results: After about 24 h in culture, an increasing number of ISC with triangular shape began to grow out from human islets. The passaged ISC expressed α -SMA, desmin, vimentin, GFAP and was positive for col- I , col-III and FN. Compared with PSC, the proliferation and migration ability of ISC was significantly slower than those of PSC. And both the human PSC and ISC were able to differentiate in vitro into adipocyte- and osteoblast-like cells. **Conclusion:** Similar to our previous rat experiment, the current study shows that human islets also contain ISC which is phenotypically similar but not identical to human PSC. Activated human ISC is a high active ECM-producing cell type, indicating human ISC may contribute to islet fibrosis in patients with T2DM.

Key words: Type 2 diabetes mellitus; T2DM; human islet; human ISC; islet stellate cell

Introduction

Type 2 diabetes mellitus (T2DM) is caused by a combination of genetic and environmental factors that result in decreased insulin function at sites of insulin action and a reduced ability of pancreatic beta cells to elevate insulin secretion in response to increased blood glucose levels. Although major progress has been achieved in our understanding of the pathogenesis of T2DM, the mechanisms underlying β -cell failure in T2DM are not fully understood.

It has been reported in the late stage of T2DM, islet fibrosis is observed in humans and in animal models [1-7], which could further aggravate β -cell failure. Several recent studies observed activated pancreatic stellate cells (PSC) in the fibrotic islets, suggesting PSC might play an important role in this process [4, 8-11]. In health, PSC is quiescent and is characterized by cytoplasmic lipid droplets rich in vitamin A. But upon in response to pancreatic injury and inflammation or exposure to inflammatory cytokines or oxidant stress, PSC can be activated, starting to proliferate, change their morphology into myofibroblast-like cells, up-regulate the expression of α -smooth

muscle actin (α -SMA), and secrete extracellular matrix (ECM) components [12].

Since the first identification of PSC, numerous *in vivo* and *in vitro* studies have provided strong evidence of a central role of PSC in pancreatic fibrogenesis associated with chronic pancreatitis and pancreatic cancer [12-19].

However, we have reported that using standard explants techniques, we isolated rat islet stellate cell (ISC) from endocrine pancreatic islets [20]. Compared with rat PSC, ISC is more rapidly activated than PSC *in vitro*. They express PSC's marker, α -SMA, vimentin, glial fibrillary acidic protein (GFAP) and is positive for ECM components col- I , col-III and FN. But, ISC differed from PSC by having reduced rates of proliferation and migration *in vitro*. Our previous indicate isolated rat islets contain a population of stellate cells which are phenotypically similar but not identical to PSC. In view of the established role of PSC in pancreatic fibrosis, we suggest that these may contribute to islet fibrosis in T2DM.

This study was designed to determine whether human islets also contain ISC, and if so, whether human ISC is different from human PSC.

Methods and materials

Human pancreatic tissue. Human pancreatic tissue was isolated from surgical samples, which was approved by the Zhongda Hospital Ethics Committee (Zhongda Hospital, Medical School, Southeast University, Jiangsu, China).

Isolation and culture of human islets. Human islets were isolated from pancreatic tissue using collagenase P. Briefly, pancreatic tissue was cut into small tissue blocks and digested with 1.5 mg/ml collagenase P (Roche) at 37 °C. Then human islets were handpicked under a stereomicroscope, as described previously [21]. The islets can be characterized by dithizone (DTZ) staining (0.1g/l, Sigma) [22]. Freshly isolated islets were subsequently cultured in cell culture dishes with 10 ml Dulbecco's modified Eagle's medium/Ham's F12 (DMEM/F12, 1:1 v/v) with 10% fetal bovine serum (FBS, Gibco) at 37°C in a 5% CO₂-air humidified atmosphere.

Isolation and culture of human PSC. Human PSC was isolated from pancreatic tissue using explant techniques [23]. Briefly, human pancreatic tissue was cut into small tissue blocks (0.5-1mm³), which was seeded into cell culture dishes in the presence of 10% FBS in DMEM/F12. Tissue blocks were cultured at 37°C in a 5% CO₂-air humidified atmosphere.

Immunostaining. Human ISC and PSC were fixed in 4% PFA in PBS for 20 min at room temperature, subsequently, immunofluorescent staining for α -SMA, desmin, vimentin, GFAP, Col-I, Col-III and FN was performed. Cells were incubated overnight at 4°C with primary antibody (Abcam, dilution rate: α -SMA desmin, vimentin and GFAP, 1:100; Col-I, Col-III and FN, 1:200), followed by a 1 h treatment with secondary antibody (Jackson ImmunoResearch Laboratories, 1:100). The sections in negative control group were incubated with PBS, instead of primary antibody. And the results indicated that the antibody and the staining worked well. Morphometric analyses were performed using Image J software.

CCK-8 assay. Cell proliferation was determined with the CCK-8 kit (Keygen Biotech). Cells were suspended at a final concentration of 5×10^3 cells/well and cultured in 96-well microplates for 24, 48 and 72 h, after which CCK-8 reagent (10 μ l) was added to each well containing 100 μ l of culture medium and the plate was incubated for 1 h at 37 °C. Viable cells were evaluated by A (A_{450nm}) using auto microplate reader (Wellscan), such that A_{450nm} was proportional to the rate of cell proliferation. All experiments were performed in quintuplicate on three separate occasions (n=15).

Cell migration assays. The transwell migration assay was performed in 24-transwell plates using inserts with a pore size of 8 μ m (Corning). 5×10^3 stellate cells were serum-starved for 24 h, after which cells were cultured in serum-free medium in the inner compartment of the transwell insert, while the plate wells which received the inserts contained DMEM/F12 supplemented with 10% FBS. The cells were incubated for 24 h at 37 °C, then fixed with 2% glutaraldehyde and stained with 1% crystal violet for 10 min. The cells that had not migrated through the membrane were removed. Cells adhering to the lower side of the insert membrane were visualized by

light microscopy and counted manually (n=5).

For the wound-healing assay, 5×10^5 cells were seeded in 6-well culture plates and grown to reach confluence. After serum starvation for 24 h, the monolayers were wounded by scraping off a strip of cells with a 10 μ L pipette tip. After 24 h cells migrating into the wound boundaries were counted manually under a phase contrast microscope (n=5).

Adipogenic and osteogenic differentiation. Adipogenic differentiation was induced by cultivation of confluent cultures in DMEM/F12 containing 20% (vol./vol.) FBS, 2.5 μ g/ml insulin, 100 μ mol/l indomethacin, 5 μ mol/l rosiglitazone and 10 nmol/l dexamethasone. For osteogenic differentiation, confluent cultures were cultivated in DMEM/F12 containing 10% (vol./vol.) FBS, 10 mmol/l β -glycerophosphate, 5 μ g/ml ascorbic acid and 10 nmol/l dexamethasone (Sigma). Cultures were maintained in differentiation media for 1 month with medium changes twice a week. Cell differentiation was analyzed by staining with Oil Red O or Alizarin Red S (Sigma) for adipogenic and osteogenic differentiation, respectively [24].

Statistical analysis. Data were presented as the means \pm SE. Statistical significance was determined by ANOVA or unpaired student's *t*-test, as appropriate, and differences between group were considered to be statistically significant when $P < 0.05$. All of the statistical analyses were performed using the Statistical Product and Services Solutions (SPSS) package (Version 11.5, SPSS Science).

Results

Isolation and culture of human ISC from isolated human islets. After about 24 h in culture, human islets adhered to the bottom of culture dishes. Then an increasing number of cells with triangular shape and large nuclei began to grow out from the edge of human islets. These cells adhered to the culture dish and assumed a stellate appearance without any detectable lipid droplets in the cytoplasm (FIG.1). We have reported previously rat islets also contained a population of stellate cells and had similar phenomenon.

Characterization of human ISC. Immunofluorescence visualization of markers for PSC (α -SMA, desmin, vimentin, GFAP) was used to characterize the human ISC at passage 5. Figure 2 showed both human PSC and ISC (a-c, PSC; d-f, ISC) were positive for α -SMA, desmin, vimentin and GFAP.

Proliferation of human ISC. To assess the rate of cell proliferation of PSC and ISC (passage 5), we used the CCK-8 assay to measure the number of viable cells during a 72 h culture period after seeding 2×10^3 cells/well in 96-well microplates, as shown in Figure 5. After 24 h, there was no significant difference between the absorbance measurements at $A_{450\text{nm}}$ in PSC and ISC groups, which indicated the number of PSC and ISC were similar at this time point. By 48 h in culture the number of PSC was significantly greater than that of ISC, and this difference was further extended after 72 h in culture, demonstrating that the ISC proliferated at a significantly slower rate than the PSC (FIG.3).

Migration of human stellate cell populations Migration was assessed by the wound-healing assay and the transwell migration assay, which are well-established in vitro systems for measuring cell motility [25, 26].

In wound healing assay, 24 h after the wound formation, the number of human ISC migrating into the cell-free area was significantly less than that of PSC (36.8 ± 7.6 % of control, $P < 0.01$, FIG. 4).

In accordance with this, ISC also showed less motility in the transwell migration assay. Thus, after 24 h the number of ISC migrating through the membrane was significantly less than that of PSC (12.3 ± 3.8 % of control, $P < 0.01$, FIG.4), which confirmed the results observed in wound healing assay.

ECM synthesis by human PSC and ISC. A panel of antibodies directed against different ECM components was used to assess the synthesis of col- I , col-III and FN by the stellate cell populations (passage 5). As shown in Figure 5, both PSC and ISC were immunopositive for col- I , col-III and FN, respectively.

Adipogenic and osteogenic differentiation. Both the human PSC and ISC were able to differentiate in vitro into adipocyte- and osteoblast-like cells, as assessed by histological staining with Oil Red O or Alizarin Red S. Moreover, as shown in Figure

6, the number of ISC differentiated into adipocyte- and osteoblast-like cells was more than that of PSC, which indicate human ISC was easier to be differentiate in vitro than PSC.

Discussion

In our previous rat research, using explant techniques, we isolated ISC exhibiting similar appearance to classical PSC from fresh isolated rat islets [20]. And immunofluorescent staining showed these out-growing cells were negative for insulin, so we excluded the possibility of β -cell out-growth. In order to distinguish these cells from PSC, we named the stellate cells migrating out from islets as ISC. The rat experiment demonstrated that rat ISC expressed PSC's marker and was immunopositive for col- I , col-III and FN, respectively. Moreover, compared with rat PSC, rat ISC was easier to be activated in vitro, and differed from PSC by having reduced rates of proliferation and migration in vitro [20].

Although we have demonstrated rat islets contained a population of stellate cells, which might contribute to the islet fibrosis in T2DM, we still have no idea whether human islets also contain these cells, and if so, whether human ISC is different from human PSC. Because T2DM has reached pandemic proportions, and current predictions show that this trend will continue [27, 28], achieving a better understanding of this complex disease is imperative. It is of great study value to figure out the answers to the above questions.

we tried to use the same method to isolate human ISC from human islets. An increasing number of ISC with triangular shapes and large nuclei migrated out from the edge of human islets in culture. These cells adhered to the culture dish and had a stellate appearance without any detectable lipid droplets in the cytoplasm, indicating ISC could be activated soon in vitro. The phenomenon we observed from the human islets was similar to what we observed in the rat experiment [20]. PSC are characterized by a range of markers including α -SMA, desmin, vimentin and GFAP [23, 29]. Here, human PSC was used as a positive control, immunofluorescent

staining for α -SMA, desmin, vimentin and GFAP was performed to identify ISC.

Both of the passaged human PSC and human ISC were immunopositive for α -SMA, desmin, vimentin and GFAP, so ISC should be included in stellate cell type.

As we discussed previously, activated PSC is an highly active ECM-producing cell type, having the capacity to synthesize and secrete large amount of ECM component such as col- I , col-III and FN. And activated PSC cannot keep the balance between MMPs and TIMPS [30], which subsequently result in the pancreatic fibrosis associated with chronic pancreatitis and pancreatic cancer [12-19, 30]. It has been reported in the late stage of T2DM, islet fibrosis is observed in humans and in animal models [1-7], which is an end stage finding associated with the extracellular matrix (ECM)/collagen and fibronectin deposition [1]. Attenuation of islet fibrosis in animal model of T2DM could protect pancreatic β -cell and improve islet function [4]. Islet fibrosis could be important in the progression of pancreatic β -cell failure in T2DM. So figuring out the mechanisms responsible for the development of islet fibrosis is of great significance to the treatment of T2DM, which could be a potential target to treat T2DM. In this study, passaged ISC was immunopositive for col- I , col-III and FN, and there was no significant difference between PSC and ISC, suggesting similar to PSC, activated human ISC should also be a highly active ECM-producing cell type. So we speculate in health, human ISC normally exist in the islet in a quiescent (non-activated) state. But upon exposure to injurious stimuli such as inflammatory cytokines, oxidant stress or hyperglycemia existing in diabetic islet, human ISC could transform to the activated state and synthesize and secrete excessive ECM, which subsequently contribute to islet fibrosis in diabetic patients.

It is interesting that similar cell was isolated from chick islets by S.P. Datar [31]. They isolated ISC from the chick islets and their results showed chick ISC was positive for desmin, vimentin and fibronectin. In their report, chick ISC could be differentiated into islet-like clusters, which was highly sensitive to l-arginine and less sensitive to glucose [31]. In contrast, our adipogenic and osteogenic differentiation experiment showed both the human PSC and ISC were capable of differentiating to adipocytes and osteocytes, it seems stellate cell may serve as pancreatic precursor cell. This idea

could be confirmed by the report from B. Davani. They described the proliferative cells from human islet as islet-derived precursor cells (hIPCs), which express CD73, CD90, and CD105, and can be differentiated in vitro into adipocytes, chondrocytes, and osteocytes. They concluded that hIPCs were a specific type of pancreas-derived mesenchymal stromal cells (MSCs). However, in addition to the MSC, our current study shows islet-derived cells also express PSC's markers. In view of PSC's function, ISC may have the potential to contribute to the islet fibrosis in T2DM.

In conclusion, similar to our previous rat experiment, the current study shows that human islets also contain ISC which is phenotypically similar but not identical to human PSC. Activated human ISC is a high active ECM-producing cell type, indicating human ISC may contribute to islet fibrosis in patients with T2DM.

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